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The Effects of NS Gene Exchange on the Pathogenicity of H5N1 HPAI Viruses in Ducks

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SUMMARY. Until 2002, H5N1 highly pathogenic avian influenza (HPAI) viruses caused only mild respiratory infections in ducks. Since then, new viruses have emerged that cause systemic disease and high mortality in ducks and other waterfowl. Studies on HPAI virus pathogenicity in ducks have been limited, and there is no clear explanation of why the pathogenicity of some H5N1 HPAI viruses has increased. The nonstructural protein 1 (NS1 protein) is known to suppress immune responses in influenza virus–infected hosts affecting virus pathogenesis. In order to determine if the NS1 protein contributes to increased virulence in ducks, single-gene reassortant viruses were generated. Exchanging the NS genes from A/Ck/HK/220/97 (a virus that produces mild disease in ducks) and A/Dk/VN/201/05 (a very virulent virus for ducks) in the rEgret/02 background (a recombinant virus derived from A/Egret/HK/757.2/02, a highly pathogenic virus in ducks) resulted in decreased mean death times compared to infection with the rEgret/02 virus in ducks, but the change was not statistically significant. Infection with the reassortant viruses affected the expression of immune-related genes in spleens and lungs when compared to controls, but when compared among them, the expression of the duck genes was similar. Furthermore, virus titers in spleen, lung, and brain as well as antigen distribution in various tissues were similar in ducks infected with the reassortant viruses. All together these data show that, under these experimental conditions, exchanging the NS gene had minimal effect on the virus pathogenicity, and it suggests that other viral genes, or combination of genes, are most likely contributing to the increased virulence of H5N1 HPAI viruses in ducks.

RESUMEN. Efectos del intercambio del gen NS en la patogenicidad de los virus H5N1 de influenza aviar de alta patogenicidad en patos.

Hasta el año 2002, el subtipo H5N1 de los virus de influenza aviar de alta patogenicidad (HPAI, por su siglas en inglés) causaron infecciones respiratorias moderadas en patos infectados. Desde esa época, nuevos virus han emergido causando enfermedad sistémica y alta mortalidad en patos y otras aves acuáticas. Los estudios sobre la patogenicidad del virus de la influenza de alta patogenicidad en patos han sido limitados, y no hay una explicación clara del porqué se ha incrementado la virulencia de algunos subtipos H5N1 del virus altamente patógeno de la influenza aviar. La proteína NS1 es conocida como causante de inmunosupresión en huéspedes infectados, afectando de esta manera la patogénesis viral. Con el fin de determinar si la proteína NS1 contribuye al incremento de la virulencia en patos, se generaron virus reacomodados genéticamente en un gene. Se realizó un intercambio de los genes NS entre el virus A/pollo/HK/220/97 (un virus que produce enfermedad moderada en patos) y el virus A/pato/VN/201/05 (un virus muy virulento para patos), utilizando como base al virus rEgret/02 (que es un virus recombinante derivado del A/garceta/HK/757.2/02, que es un virus de alta patogenicidad de los patos). Este intercambio resultó en la disminución de los tiempos de mortalidad promedio de los patos en comparación con el virus rEgret/02, pero el cambio no fue estadísticamente significativo. La infección con el virus reacomodado afectó la expresión de los genes relacionados con el sistema inmune en el bazo y el pulmón, comparada con los controles, pero cuando se realizó la comparación entre ellos, la expresión de los genes del pato fue similar. Aún así, los títulos del virus en bazo, pulmón y cerebro, así como la distribución de antígenos en varios tejidos, fue similar en patos infectados con los virus reacomodados. Estos datos juntos muestran que bajo estas condiciones experimentales, el intercambio del gen NS tuvo un efecto mínimo en la patogenicidad de los virus, y sugiere que es más probable que otros genes virales, o combinaciones de genes contribuyan al incremento de la virulencia del subtipo H5N1del virus de HPAI en patos.

Key words: H5N1, avian influenza, NS1, ducks, gene expression

Abbreviations: BHI = brain heart infusion; CEF = chicken embryo fibroblast; CPE = cytopathic effect; dpi = day post-inoculation; ECE = embryonating chicken egg; EID_{50} = 50% egg infective dose; HPAI = highly pathogenic avian influenza; IFN = interferon; IN = intranasally; MDT = mean death time; NCBI = National Center for Biotechnology Information; NS1 = nonstructural protein 1; RT-PCR = reverse transcription-polymerase chain reaction; $TCID_{50}$ = 50% tissue culture infectious dose

Ducks and other waterfowl are natural reservoirs of all subtypes of influenza A virus (24). Prior to 2002, infection of ducks with H5N1 highly pathogenic avian influenza (HPAI) viruses resulted in local virus replication in lungs and associated mild respiratory lesions (13,14). Since then, new strains have been identified that cause severe systemic disease and high mortality in ducks (4,12,15,21,22). Experimentally, these viruses reach high titers in multiple organs, especially in the respiratory tract, heart, and brain (7,15,22). Until now there has been no clear explanation of why the pathogenicity of H5N1 HPAI viruses has increased. Reassortment of viral genes due to coinfection of ducks may contribute to this increased in virulence (20). Therefore it is important to study individual viral genes to determine their role in the pathogenesis of HPAI viruses. Previous studies showed that changes in the polymerase genes, PA and PB1, and in the HA gene are associated with high pathogenicity of H5N1 viruses in mallard ducks (6,23). The nonstructural protein 1 (NS1 protein) of avian influenza viruses may also have a role in the pathogenicity of these viruses because it has the ability to suppress

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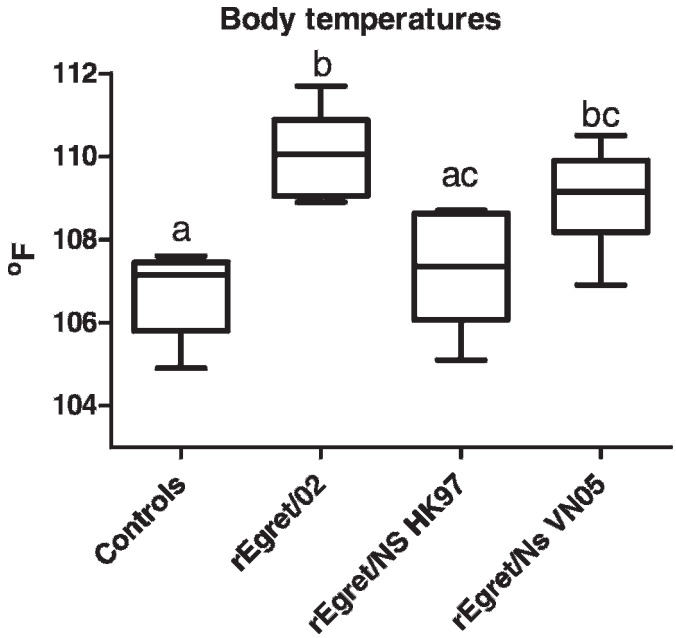

Fig. 1. Comparison of body temperatures of 2-wk-old ducks inoculated with H5N1 HPAI viruses, 3 dpi. Ducks were infected intranasally with 10^6 EID_{50} of the H5N1 HPAI viruses. Groups with different lowercases letter are significantly different ($P \leq 0.05$).

interferon (IFN) and other immune-related genes, as shown in mammalian systems (5,19) and in chickens (3,9). Until now it has not been clear whether the NS1 protein has the same effect on the immune response of ducks to infection or if differences in NS1 protein could be the cause of increased virulence observed with H5N1 HPAI viruses in ducks.

Previous studies showed that the A/Ck/HK/220/97 H5N1 HPAI virus produced mild respiratory disease in ducks (14) whereas A/Dk/VN/201/05 H5N1 HPAI virus produced severe disease and death of ducks (15). Amino acid sequence analyses revealed that the NS1 protein of A/Ck/HK/220/97 has a glutamic acid at position 92. Recent studies demonstrated that the NS1 protein of H5N1 viruses carrying a glutamic acid at position 92 are resistant to the antiviral effect of interferons and tumor necrosis factor α in pigs (10,18). In contrast the NS1 protein of the A/Dk/VN/201/05 has a 5 amino acid deletion from position 80 to 84 and has residues Phe103 and Met106. Residues Phe103 and Met106 of the NS1 protein of influenza viruses have been associated with prevention of IFN-induced antiviral state *in vitro* (8). A/Ck/HK/220/97 and A/Dk/VN/201/05 both carry an alanine at position 149, which has been correlated with the ability of avian influenza viruses to antagonize IFN induction in chicken embryo fibroblasts (CEFs) (9). Our preliminary studies revealed that infection of duck embryo fibroblasts with these viruses resulted in differences in the level of expression of immune-related genes, especially IFN-α and MX1 genes (unpubl. data).

The objective of this study was to determine the role of the NS1 protein in the pathogenicity of HPAI H5N1 viruses in ducks. To this end, single-gene reassortant viruses were generated. The NS gene from A/Ck/HK/220/97 or A/Dk/VN/201/05 was exchanged in the rEgret/02 background, a recombinant virus derived from A/Egret/HK/757.2/02, which is highly pathogenic in ducks. Clinical signs, lesions, virus tissue distribution, virus load in tissues, expression of host genes, and nitric oxide levels in tissues in response to infection were examined.

MATERIALS AND METHODS

Generation of infectious reassortant viruses. A highly pathogenic recombinant H5N1 HPAI virus was derived from A/Egret/Hong Kong/757.2/02 as follows and is referred to as rEgret/02. RNA was extracted from virus stocks of the wild-type virus after propagation in embryonating chicken eggs (ECEs) using Trizol LS (Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's instructions. Transcription plasmids were constructed as previously described (11). Next 293T cells were transfected with 1 μg of each of the eight transcription and four protein expression plasmids and 11 μl of Lipofectamine 2000 (Invitrogen) in a 2 ml volume of OptiMEM I (Invitrogen). After 72 hr, ECEs were inoculated with 100 μl of the cell supernatant. Virus was harvested from the allantoic fluid of eggs 36–48 hr after inoculation and titrated in ECEs. Titration end points in ECEs were calculated by the method of Reed and Muench (16). In addition to the reconstitution of the parental strain by reverse genetics, two single-gene reassortants containing the NS gene of either A/Ck/HK/220/97 (rEgret/NS HK97) or A/Dk/VN/201/05 (rEgret/NS VN05) in the rEgret/02 backbone were generated. All rescued viruses were sequenced to confirm that the genes were correct. The nucleotide sequences of A/Egret/Hong Kong/757.2/02, A/Ck/HK/220/97, and A/Dk/VN/201/05 are available from GenBank (National Center for Biotechnology Information [NCBI]) under accession numbers AY676022, AY676026, AY676030, AY676034, AY676038, AY676042, AY676046, AY676050 (A/Egret/Hong Kong/757.2/02), AF046080–AF046087(A/Ck/HK/220/97), and EU930884–EU930891(A/Dk/VN/201/05). The nucleotide sequences of rEgret/02 is identical to A/Egret/Hong Kong/757.2/02. All experiments using H5N1 HPAI viruses were conducted in biosafety level 3 (BSL-3) enhanced facilities at Southeast Poultry Research Laboratory, ARS/USDA, in Athens, GA (2).

***In vivo* characterization of reassortant viruses.** Two-week-old Pekin ducks (*Anas platyrhynchos*) obtained from a commercial hatchery were used for determining the pathogenicity of the three H5N1 HPAI viruses. Serum samples were collected from a representative number of ducks prior to inoculation to ensure that the birds were serologically negative for AIV. Ducks were housed in self-contained isolation units (Mark 4, Controlled Isolation Systems, San Diego, CA) that were ventilated under negative pressure with HEPA-filtered air and maintained under continuous lighting. Feed and water were provided

Table 1. Primer sequences for semiquantitative RT-PCR assays.

Gene[A]	5′ → 3′ Upstream primer	5′→ 3′ Downstream primer	GenBank acc. no.
MHC II	AGACCAAGGGGTTCTTCCAT	AAAAGCCCGTCACGTAACAC	AF390589
IL-8	GCAGGCACACAGACTCTGAA	TTGGCCAGAATTGCCTTTAC	AB236335
IL-18	AAATCCTCCATCGCTTCCTT	TTTCCCGTGTTCTTCCTCAC	DQ522948
MX1	ACGCTCTACCAAGGCAGAAA	GCTTTGCTGAGCCGATTAAC	Z21550
IFN-α	TCCTCCAACACCTCTTCGAC	GGGCTGTAGGTGTGGTTCTG	DQ861429
MIP1-α	CTGCCTGAGGACGAGCGAGAATC	TGGGTGGCAGAGGGGAGAAGA	AY682098
β-actin	CCCCCGTGCTGTGTTCCCATCTATCG	GGGTGCTCCTCAGGGGCTACTCTCAG	EU309690.1

[A]IFN-α, interferon alpha; IL-8, interleukin 8; IL-18, interleukin 18; MHC II, major histocompatibility complex class II; MIP1-α, macrophage inflammatory protein 1 alpha; MX1, myxovirus resistance gene 1.

Table 2. Morbidity, mortality, and MDT resulting from intranasal inoculation of 2-wk-old ducks with 10^6 EID_{50} of the H5N1 HPAI viruses.

Virus	Days post-inoculation[A]									MDT (days)
	1	2	3	4	5	6	7	8	9	
rEgret/02	0/0/8	1/0/8	1/0/8	7/1/8	4/3/7	1/2/4	1/1/2	1/0/1	1/0/1	5.4
rEgret/NS HK97	1/1/8	0/1/7	2/0/6	4/2/6	1/3/4	1/0/1	0/1/1	–	–	4.1
rEgret/NS VN05	0/0/8	0/1/8	4/0/7	5/2/7	2/3/5	1/1/2	1/0/1	1/0/1	1/0/1	4.4

[A]Number of sick ducks/number of dead ducks/total ducks; – = no ducks remaining.

with *ad libitum* access. The experimental design has been previously described (12). Briefly, ducks were separated into a control group and three virus-inoculated groups. Control birds were intranasally (IN) inoculated with 0.1 ml of the sham inoculum consisting of sterile allantoic fluid diluted 1:300 in brain heart infusion medium. The three virus-inoculated groups were inoculated IN with 0.1 ml of an inoculum containing 10^6 50% egg infective doses (EID_{50}) of one of the viruses. Four birds from each group containing 12 birds were euthanatized and necropsied at 2 days post-inoculation (dpi). Gross lesions were recorded. Trachea, lung, bursa, kidney, thymus, brain, liver, heart, pancreas, intestine, spleen, tongue, harderain gland, skin, and thigh muscle tissues from two ducks per group were collected in 10% neutral buffered formalin solution. The remaining ducks were evaluated for clinical signs for 10 days. Mean death time (MDT) was calculated by determining the sum of the day of death for the ducks and dividing it by the total number of dead ducks. Body temperatures were taken at 3 dpi. Sample birds, moribund birds, and all birds remaining at the end of the experiment were euthanatized by the intravenous administration of sodium pentobarbital (100 mg/kg body weight).

Virus isolation and titrations. Portions of the spleens, lungs, and brains from the four birds per group necropsied at 2 dpi were collected in brain heart infusion medium (BHI; BD Bioscience, Sparks, MD) and stored frozen at −70 C until use. For virus titration, tissues were homogenized (10% [wt/vol]) and diluted in BHI. Infectious virus titers were determined by cytopathic effect (CPE) in duck fibroblasts (cell line CCL-141) and reported as 50% tissue culture infectious dose ($TCID_{50}$). The $TCID_{50}$ assay end point was calculated by the method of Reed and Muench (16).

Histopathology and immunohistochemistry. Tissues samples from two ducks per group fixed by submersion in 10% neutral buffered formalin were routinely processed and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin. Duplicate sections were cut and immunohistochemically stained using a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus nucleoprotein antigen. The procedures used to perform the immunohistochemistry followed those previously described (13).

RNA extraction. Total cellular RNA for the evaluation of gene expression was prepared from spleen or lung tissues collected from four ducks per group 2 dpi. Tissue samples were homogenized in 3 ml of Eagle's minimal essential medium, Alpha 1× (Invitrogen), by passing the tissues through 100-μm cell strainers (BD Bioscience), added to 6 ml Trizol, inverted, and stored at −80 C. Chloroform (1.2 ml) was added and spun, and the aqueous phase was added to an equal volume of 70% ethanol. A Qiagen RNA midiprep kit (Qiagen, Valencia, CA) was used to isolate the RNA from the aqueous phase-ethanol solution.

Reverse transcription-PCR (RT-PCR) and sequencing. The presence of duck MHC II, IL-8, IL-18, MX1, IFN-α, MIP1-α, and β-actin mRNAs were determined using the Qiagen One step RT-PCR enzyme kit (Qiagen) according to manufacturer's instructions. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). All primers were designed based on the NCBI nucleotide sequences for these genes (Table 1). The reaction was carried out in a 50 μl final volume reaction using 100 ng of pooled cellular RNA from four birds per group. The PCR products were analyzed on 1.5 % agarose gel and used directly for sequencing. Sequencing was performed with an ABI 3730XL DNA analyzer. Bands were quantified using the Image Processing and Analysis in Java (http://rsb.info.nih.gov/ij/) and normalized to β-actin control. Gene expression in tissues of sham-inoculated ducks was arbitrarily set to one.

Nitric oxide assays. Total nitrate and nitrite content produced by nitric oxide synthase was measured in serum samples at 2 dpi. Serum was passed through a 10 kDa ultrafilter (Millipore Corp., Billerica, MA) prior to analysis. Total protein in the filtered serum was measured using a DC Protein Assay (Bio-Rad, Hercules, CA). A Nitric Oxide Synthase

Table 3. Distribution of viral antigen in tissues from 2-wk-old ducks inoculated with H5N1 HPAI viruses. Tissues were collected at 2 dpi and immunohistochemically stained with antibodies to avian influenza virus nucleoprotein.[A]

Tissue	rEgret/02		rEgret/NS HK97		rEgret/NS VN05	
	Duck 1	Duck 2	Duck 1	Duck 2	Duck 1	Duck 2
Trachea	++	+	++	++	++	++
Lung	++	+	++	+	++	+++
Heart	+	+++	++	++	+++	++
Brain	++	+++	++	++	+++	+++
Pancreas	+	++	+	++	+	+++
Intestine	−	−	−	−	−	+
Liver	+	−	−	−	−	−
Kidney	−	−	−	−	+	−
Spleen	+	+	+	+	+	++
Bursa	−	−	+	−	+	−
Thymus	+	++	+	+	+	++
Muscle	+	++	−	−	+	++
Tongue	+	++	+	−	+	++
Harderian gland	−	−	+	−	++	−
Feather	+	++	++	+	+++	+++

[A]Immunohistochemical staining: − = no antigen staining; + = infrequent; ++ = common; +++ = widespread staining.

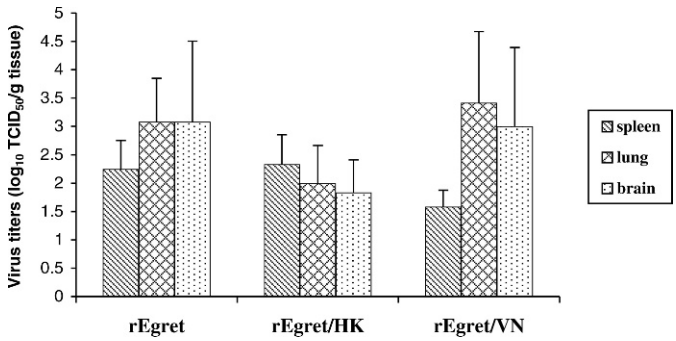

Fig. 2. Virus titers in spleen, lung and brain tissues from ducks infected with H5N1 HPAI viruses, at 2 dpi. Infectious virus titers were determined by CPE in duck fibroblasts (cell line CCL-141) and reported as $TCID_{50}$. Each data point represents the mean and range virus titer (log_{10} $TCID_{50}$/gram of tissue) from four ducks.

Assay kit (Calbiochem, Gibbstown, NJ) was used according to the manufacturer's specifications on 40 μl of filtered serum. Values were normalized to the total protein in the filtered serum.

NS1 sequence comparison. The NS1 amino acid sequences of A/Ck/HK/220/97, A/Dk/VN/201/05, and A/Egret/HK/757.2/02 were aligned using the algorithm Clustal V (Lasergene 7.0; DNAStar, Madison, WI).

Statistical analyses. Data were analyzed using Prism v. 5.01 software (GraphPad Software, Inc., San Diego, CA), and values are expressed as the mean ± SEM. The one-way ANOVA with Tukey-Kramer post-test was used to analyze the data. Statistical significance was set at $P < 0.05$.

RESULTS

Clinical signs, morbidity and mortality. All ducks inoculated with the recombinant viruses presented moderate to severe depression and anorexia. Several ducks infected with the viruses displayed mild to severe neurological signs beginning at 2 dpi and characterized by tremors, uncontrollable shaking, marked loss of balance, lack of coordination, tilted head, seizures, and paralysis. The onset of neurological signs was earlier and signs more severe in ducks inoculated with rEgret/NS VN05. Body temperatures taken at 3 dpi demonstrated that the ducks inoculated with rEgret/02 or rEgret/NS VN05 had fever that was significantly higher than that observed in controls or ducks inoculated with rEgret/NS HK97 (Fig. 1). The morbidity and mortality resulting from infection with the viruses were recorded and are summarized in Table 2. Infection of ducks with rEgret/NS HK97 virus resulted in 8 out of 8 ducks dying and a MDT of 4.1, whereas infection with rEgret/NS VN05 virus resulted in a mortality of 7 of 8 ducks and a MDT of 4.4. Infection with the rEgret/02 virus resulted in a mortality of 7 of 8 ducks and a MDT of 5.4 days. However, the differences observed in MDT were not significant.

Microscopic lesions, antigen distribution, and virus titers in tissues. Ducks inoculated with the recombinant viruses presented similar histological lesions in tissues at 2 dpi including diffuse interstitial pneumonia in the lung, moderate tracheitis, pancreatitis, moderate cardiac degeneration, and multifocal nonsupurative encephalitis, among others. The distribution of avian influenza antigen staining in tissues is summarized in Table 3. Viral antigen staining was present in multiple organs, but mostly found in the heart and in the brain, followed by the trachea and lungs, and was more widespread in tissues from ducks infected with rEgret/NS VN05. AIV antigen was present in tissue macrophages, cardiac and skeletal muscle myocytes, neurons and glial cells in the brain, pancreatic accinar cells, respiratory epithelium of trachea, and adrenal corticotrophic cells. The virus titers of rEgret/NS VN05 were higher in the lung, especially when compared with the rEgret/NS HK97, but were decreased in the spleen when compared to rEgret/02 and rEgret/NS HK97 (Fig. 2). Overall, the virus that least replicated in tissues was the rEgret/NS HK97.

Expression of immune-related genes. Differences in the expression of immune-related genes in spleens and lungs of virus-infected ducks were investigated. MX1 and MIP1-α genes expression in both spleens and lungs was clearly up-regulated by infection with the recombinant viruses when compared to the sham-inoculated ducks (Fig. 3). In contrast, the level of expression of MHC II in spleens and lungs was decreased. However, the expression of these genes was similar when the viruses were compared among themselves. IFN-α, IL-8, and IL-18 were expressed similarly in tissues from the ducks infected with the recombinant viruses and the

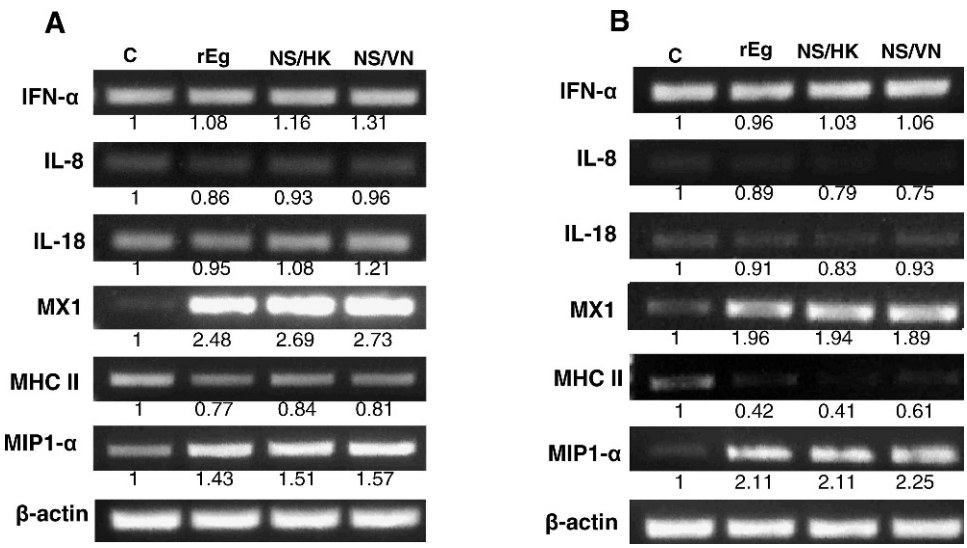

Fig. 3. Expression of immune-related genes in spleens (A) and lungs (B) of ducks infected with rEgret/02, rEgret/NS HK97, or rEgret/NS VN05. Measurements of the intensity of the bands are indicated below each band. (C) Sham-inoculated controls; rEg, rEgret/02; NS/HK, rEgret/NS HK97; NS/VN, rEgret/NS VN05; IFN-α, interferon alpha; IL-8, interleukin 8; IL-18, interleukin 18; MX1, myxovirus resistance gene 1; MHC II, major histocompatibility complex class II; MIP-α, macrophage inflammatory protein 1 alpha.

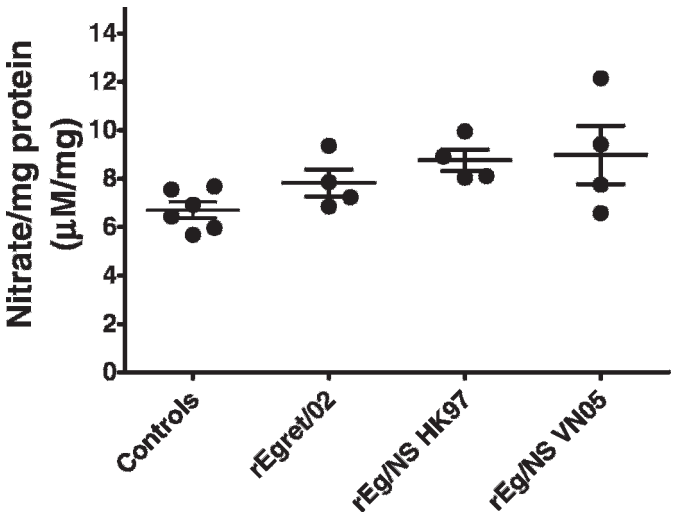

Fig. 4. Total nitrate and nitrite content in serum samples from ducks. Serum was obtained from blood collected at 2 days post-inoculation. Values are the mean ± SEM ($n = 4$), and values were standardized to the amount of total protein in the filtered serum.

controls, although IFN-α and IL-18 were slightly up-regulated in spleens of the ducks infected with rEgret/NS VN05.

Nitric oxide levels in spleens. There is evidence that nitric oxide has antiviral properties (1). In our study the levels of nitric oxide in spleens were higher as a result of infection with the recombinant viruses when compared to the controls, especially in spleens infected with rEgret NS/VN05 (Fig. 4). However, these differences were not statistically significant.

NS gene sequence comparison. Sequence comparison of A/Egret/HK/757.2/02 and A/Ck/HK/220/97 showed that the NS gene segments shared 80.6% nucleotide identity resulting in 19 amino acid differences (Fig. 5). Comparison of A/Egret/HK/757.2/02 and A/Dk/VN/201/05 showed 93.4% sequence identity with 10 amino acid differences. Comparison of A/Ck/HK/220/97 and A/Dk/VN/201/05 revealed 79.1% nucleotide sequence identity and 25 amino acid changes differentiating the NS1 amino acid sequences.

DISCUSSION

The pathogenicity of H5N1 HPAI viruses in ducks has increased in the past years, with viruses causing severe disease and high mortality. The cause of the increased virulence of these viruses has not been determined. One possible explanation is the generation of new viruses by reassortment of viral genes due to coinfection. To investigate the role of the NS1 protein in the pathogenicity of HPAI H5N1 viruses in ducks we used reverse genetics to generate H5N1 reassortant viruses that share seven genes and differ from each other only in the NS gene. This study demonstrated that substitution of the NS gene between viruses that produce different diseases in ducks had little impact on the outcome of disease. Substituting the rEgret/02 NS gene with the NS from A/Dk/VN/201/05 decreased the MDT 1 day. This was expected since infection of ducks with the wild-type DK/VN/201/05 causes severe disease with 100% mortality in only 3.4 days (15). Intriguingly, substituting the NS in the rEgret/02 backbone with the NS gene from A/Ck/HK/220/97 also decreased the MDT one day. Because infection of ducks with the A/Ck/HK/220/97 is known to cause only mild clinical disease and no mortality (14), an increase in the MDT would be expected if the NS from this virus preserved the original characteristics of the wild-type virus. Instead, the decrease in the MDT suggests that the substitution of the NS may have increased the compatibility among the eight genes, making this virus slightly more pathogenic.

All viruses studied produced similar clinical signs and histopathological lesions; however, neurological signs were more severe and were manifested earlier in ducks infected with rEgret/NS VN05. Viral antigen staining was also more widespread in tissues from these birds, and higher virus titers were observed in lungs when compared to the other two viruses. This is similar to what is observed when ducks are infected with the wild-type A/Dk/VN/201/05 (15), and exchanging the NS may have contributed to these events.

Several studies reported that the NS1 protein affects virus pathogenicity by modulating IFN and other immune-related host genes (3,5,9). In avian cells a recent study showed that IFN-stimulated genes (ISG12, LY6E, and haemopoiesis-related membrane protein 1 gene) were up-regulated following infection with a HPAI virus (25). Avian influenza viruses have also been shown to

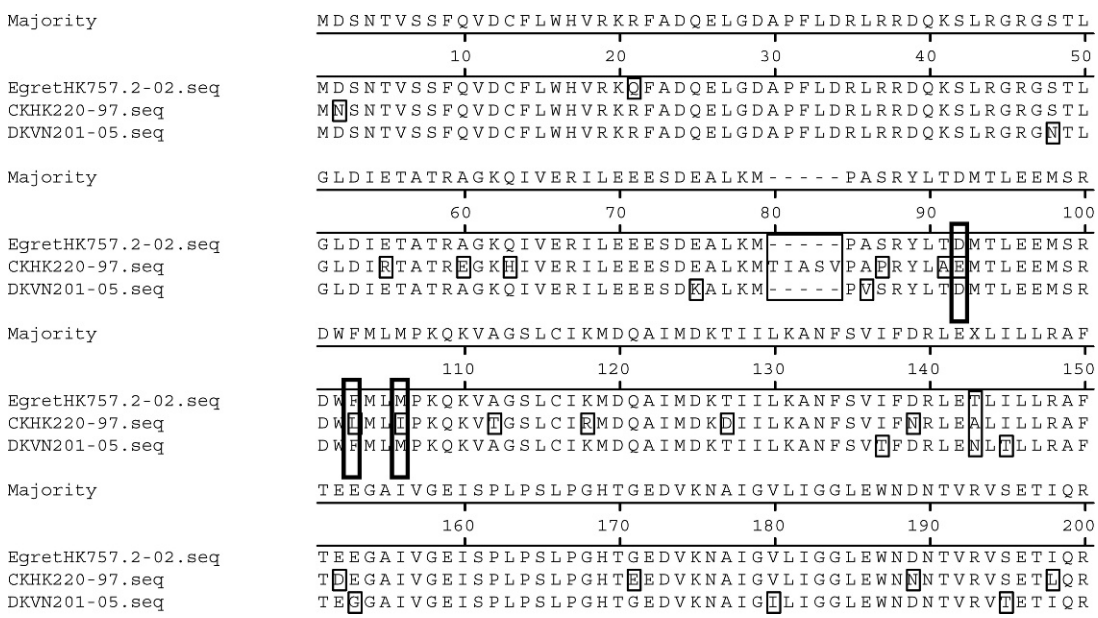

Fig. 5. Amino acid differences between the NS1 sequences of A/Ck/HK/220/97, A/Dk/VN/201/05, and A/Egret/HK/757.2/02.

antagonize IFN-α/β production in CEFs and in chickens (3,9). Our own studies demonstrated that HPAI viruses have varied ability to regulate the expression of host genes in CEFs (17). In the present study, substituting the NS genes induced changes in the level of immune-related gene expression, nevertheless at similar levels. Infection with the recombinant viruses had little effect on the expression of IFN-α, IL-8, or IL-18. The expressions of MX1 and MIP1-α increased, and the expression of MHC II decreased in tissues of virus-infected ducks, but at similar levels. In this study IFN-α gene expression did not correlate with MX1 gene expression. A reason for this could be the time point at which the expression was measured, IFN-α possibly being expressed earlier than MX1, and the increase missed at the time point measured. As for all the genes mentioned, it is clear that more information is needed on the role of IFN-α and MX1 in response to viral infection in ducks. The results of this study suggest that either the NS1 proteins of these viruses regulated these particular host genes in the same manner, or other viral proteins are driving this regulation. In addition, infection with the viruses resulted in a slight increase in the levels of nitric oxide in spleens. Although these increases were at different levels, no correlation between nitric oxide levels and virus replication or outcome of infection was observed.

Although the NS1 proteins of the viruses that we chose for this study had amino acid residues reported as important in the immune response modulation and/or in the outcome of disease in chickens and mammals, we found that at least in this particular case the exchange of the NS gene was not enough to induce a significant change in pathogenicity of the parental virus in ducks, suggesting the importance of other viral genes or combination of genes, including NS1, in the pathogenicity of these viruses in ducks.

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